

## Claims

1. An isolated and purified nucleic acid, encoding an *Aspergillus ochraceus* 11 alpha hydroxylase.
2. An isolated and purified nucleic acid of claim 1, wherein said nucleic acid comprises the DNA sequence of SEQ ID NO: 1.
3. An isolated and purified *Aspergillus ochraceus* 11 alpha hydroxylase.
4. An isolated and purified *Aspergillus ochraceus* 11 alpha hydroxylase of claim 3 comprising an amino acid sequence of SEQ ID NO: 2.
5. A fusion protein comprising the amino acid sequence of the *Aspergillus ochraceus* 11 alpha hydroxylase of claim 4.
6. An isolated and purified nucleic acid, encoding an *Aspergillus ochraceus* oxidoreductase.
7. An isolated and purified nucleic acid of claim 6, wherein said nucleic acid comprises the DNA sequence of SEQ ID NO: 5.
8. An isolated and purified *Aspergillus ochraceus* oxidoreductase.
9. An isolated and purified *Aspergillus ochraceus* oxidoreductase of claim 8 comprising an amino acid sequence of SEQ ID NO: 6.
10. An isolated and purified nucleic acid encoding an enzyme that can catalyze the 11 alpha hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); and 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids).
11. An isolated and purified nucleic acid of claim 10, wherein said enzyme does not catalyze the 15 beta hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; and 3 keto delta 6, 7 steroids.
12. The isolated and purified nucleic acid of claim 10 or claim 11, wherein said hydroxylation is selected from the group

consisting of:

- (a) canrenone to 11 alpha hydroxy canrenone;
- (b) androstenedione to 11 alpha hydroxy androstenedione;
- 5 (c) aldona to 11 alpha hydroxy aldon;
- (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD;
- (e) mexrenone to 11 alpha hydroxy mexrenone;
- (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone;
- 10 (g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone;
- (h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone;
- 15 (i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone;
- (j) testosterone to 11 alpha hydroxy testosterone;
- (k) progesterone to 11 alpha hydroxy progesterone;
- (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and
- 20 (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone.

13. The isolated and purified nucleic acid of claim 12, wherein said hydroxylation is selected from the group consisting of:

- (a) canrenone to 11 alpha hydroxy canrenone;
- (b) androstenedione to 11 alpha hydroxy androstenedione;

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(c) aldona to 11 alpha hydroxy aldona; and

(d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD.

14. The isolated and purified nucleic acid of claim 13, wherein said hydroxylation is from canrenone to 11 alpha hydroxy canrenone.

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15. A method of expressing a protein that can catalyze the 11 alpha hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; 3 keto delta 6, 7 steroids; and 3 keto delta 1, 2 delta 4, 5 steroids comprising;

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(a) transforming or transfecting host cells with an expression cassette comprising a promoter operably linked to a nucleic acid that encodes said protein, and

(b) expressing said protein in said host cells.

16. A method of producing the protein of claim 15, further comprising the step of recovering said protein.

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17. The method of claim 15 or claim 16 wherein said protein is an *Aspergillus ochraceus* 11 alpha hydroxylase.

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18. The method of claim 17, further comprising expressing an electron donor protein, wherein said electron donor protein can donate electrons to said protein that can catalyze the 11 alpha hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; 3 keto delta 6, 7 steroids; and 3 keto delta 1, 2 delta 4, 5 steroids.

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19. The method of claim 18 wherein said electron donor protein is selected from the group consisting of human oxidoreductase and *Aspergillus ochraceus* oxidoreductase.

20. The method of claim 18 wherein said electron donor protein is *Aspergillus ochraceus* oxidoreductase.

21. The method of claim 18, wherein the nucleic acid encoding said 11 alpha hydroxylase and said electron donor protein are on separate expression cassettes.

5 22. The method of claim 18, wherein the nucleic acid encoding said 11 alpha hydroxylase and said electron donor protein are on the same expression cassettes.

10 23. The method of claim 21 wherein said 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is human oxidoreductase.

15 24. The method of claim 22 wherein said 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is human oxidoreductase.

20 25. The method of claim 21 wherein said 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is *Aspergillus ochraceus* oxidoreductase.

25 26. The method of claim 22 wherein said 11 alpha hydroxylase is *Aspergillus ochraceus* 11 alpha hydroxylase and said electron donor protein is *Aspergillus ochraceus* oxidoreductase

27. The method of claim 17, wherein said *Aspergillus ochraceus* 11 alpha hydroxylase is SEQ ID NO: 2.

30 28. The method of claim 19, wherein said human oxidoreductase is SEQ ID NO: 4.

29. The method of claim 19, wherein said *Aspergillus ochraceus* oxidoreductase is SEQ ID NO: 6.

30. An isolated and purified polypeptide that can catalyze the 11 alpha hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); and 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids).

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31. An isolated and purified polypeptide claim 30, wherein said enzyme does not catalyze the 15 beta hydroxylation of a compound

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selected from the group consisting of: 3 keto delta 4,5 steroids; 3 keto delta 4, 5 delta 6, 7 steroids; and 3 keto delta 6, 7 steroids.

32. The isolated and purified polypeptide of claim 30 or claim

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31, wherein said hydroxylation is selected from the group consisting of :

(a) canrenone to 11 alpha hydroxy canrenone;

(b) androstenedione to 11 alpha hydroxy androstenedione;

(c) aldona to 11 alpha hydroxy aldona;

(d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD;

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(e) mexrenone to 11 alpha hydroxy mexrenone;

(f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone;

(g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone;

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(h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone;

(i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone;

(j) testosterone to 11 alpha hydroxy testosterone;

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- (k) progesterone to 11 alpha hydroxy progesterone;
- (l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone; and
- (m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone.

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- 33. The isolated and purified polypeptide of claim 32, wherein said hydroxylation is selected from the group consisting of:
  - (a) canrenone to 11 alpha hydroxy canrenone;
  - (b) androstenedione to 11 alpha hydroxy androstenedione;
  - (c) aldonia to 11 alpha hydroxy aldonia; and
  - (d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD.

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- 34. The isolated and purified enzyme of claim 33, wherein said hydroxylation is from canrenone to 11 alpha hydroxy canrenone.

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- 35. An expression cassette comprising a promoter operably linked to an isolated and purified nucleic acid of claim 10 encoding a polypeptide that can catalyze the 11 alpha hydroxylation of a compound selected from the group consisting of: 3 keto delta 4,5 steroids (3 keto delta 4 steroids); 3 keto delta 4, 5 delta 6, 7 steroids (3 keto delta 4 delta 6 steroids); 3 keto delta 6, 7 steroids (3 keto delta 6 steroids); and 3 keto delta 1, 2 delta 4, 5 steroids (3 keto delta 1 delta 4 steroids).

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- 36. An expression cassette comprising a promoter operably linked to an isolated and purified nucleic acid of claim 6 encoding *Aspergillus ochraceus* oxidoreductase.

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- 37. An expression cassette of claim 36 wherein said nucleic acid is SEQ ID NO: 06.

38. An expression cassette comprising a heterologous DNA encoding an enzyme from the metabolic pathway for the synthesis of sitosterol to eplerenone wherein said enzyme catalyzes at least one conversion selected from the group consisting of:

5 (a) canrenone to 11 alpha hydroxy canrenone;

(b) androstenedione to 11 alpha hydroxy androstenedione;

(c) aldona to 11 alpha hydroxy aldona;

(d) ADD (1,4 androstenedienedione) to 11 alpha hydroxy ADD;

(e) mexrenone to 11 alpha hydroxy mexrenone;

10 (f) 6 beta mexrenone to 11 alpha hydroxy 6 beta mexrenone;

(g) 9 alpha mexrenone to 11 alpha hydroxy 9 alpha mexrenone;

(h) 12 beta mexrenone to 11 alpha hydroxy 12 beta mexrenone;

(i) delta 12 mexrenone to 11 alpha hydroxy delta 12 mexrenone;

15 (j) testosterone to 11 alpha hydroxy testosterone;

(k) progesterone to 11 alpha hydroxy progesterone;

(l) mexrenone 6,7-bis-lactone to 11 alpha hydroxy mexrenone 6,7-bis-lactone;

(m) mexrenone 7,9-bislactone to 11 alpha hydroxy mexrenone 7,9-bislactone;

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and wherein the heterologous DNA is operably linked to control sequences required to express the encoded enzymes in a recombinant host.

25 39. The expression cassette according to claim 38 , characterized in that the heterologous DNA coding sequences are selected from the group consisting of the following genus and species: *Aspergillus ochraceus*, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Streptomyces*

fradiae, *Bacillus megaterium*, *Pseudomonas cruciviae*,  
*Trichothecium roseum*, *Fusarium oxysporum* *Rhizopus arrhizus*,  
*Absidia coerula*, *Absidia glauca*, *Actinomucor elegans*, *Aspergillus*  
5 *flavipes*, *Aspergillus fumigatus*, *Beauveria bassiana*,  
*Botryosphaeria obtusa*, *Calonectria decora*, *Chaetomium cochliodes*,  
*Corynespora cassiicola*, *Cunninghamella blakesleeana*,  
*Cunninghamella echinulata*, *Cunninghamella elegans*, *Curvularia*  
10 *clavata*, *Curvularia lunata*, *Cylindrocarpon radicicola*, *Epicoccum*  
*humicola*, *Gongronella butleri*, *Hypomyces chrysospermus*,  
*Monosporium olivaceum*, *Mortierella isabellina*, *Mucor mucedo*,  
*Mucor griseocyanus*, *Myrothecium verrucaria*, *Nocardia corallina*,  
15 *Paecilomyces carneus*, *Penicillium patulum*, *Pithomyces*  
*atroolivaceus*, *Pithomyces cynodontis*, *Pycnosporium* sp.,  
*Saccharopolyspora erythrae*, *Sepedonium chrysospermum*,  
*Stachyliodium bicolor*, *Streptomyces hygroscopicus*, *Streptomyces*  
20 *purpurascens*, *Syncephalastrum racemosum*, *Thamnostylum*  
*piriforme*, *Thielavia terricola*, and *Verticillium theobromae*,  
*Cephalosporium aphidicola*, *Cochliobolus lunatas*, *Tieghemella*  
*orchidis*, *Tieghemella hyalospora*, *Monosporium olivaceum*,  
25 *Aspergillus ustus*, *Fusarium graminearum*, *Verticillium glaucum*,  
and *Rhizopus nigricans*.

40. The expression cassette according to claim 39, wherein the genus and species are selected from the group consisting of *Aspergillus ochraceus*, *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Streptomyces fradiae*, *Bacillus megaterium*, *Pseudomonas cruciviae*, *Trichothecium roseum*, *Fusarium oxysporum* *Rhizopus arrhizus*, and *Monosporium olivaceum*.

30 41. The expression cassette according to claim 40, wherein the genus species is *Aspergillus ochraceus*.

42. A recombinant host cell and progeny thereof comprising at least one expression cassette according to claim 38.

35 43. A process for making one or more enzymes from the metabolic pathway for the synthesis of sitosterol to

eplerenone comprising incubating the recombinant host cell of claim 42 in a nutrient medium under conditions where the one or more enzymes encoded by the heterologous DNA are expressed and accumulate.

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44. A process for the selective oxidation of a compound to an hydroxylated product, which process comprises the steps of: (a) incubating the compound to be hydroxylated in the presence the recombinant host cells of claim 42 under conditions where the compound is hydroxylated and the hydroxylated product accumulates, and (b) recovering the hydroxylated product.

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45. A process for the selective hydroxylation of a compound to an hydroxylated product in vitro, which process comprises the steps of: (a) incubating the compound to be hydroxylated in the presence of the enzymes produced in the process of claim 44 under conditions where the compound is hydroxylated and the hydroxylated product accumulates, and (b) recovering the hydroxylated product.

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46. A host cell harboring an expression cassette of any of claim 35.

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47. A host cell of claim 46, wherein said expression cassette is integrated into the chromosome of said host cell.

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48. A host cell of claim 46, wherein said expression cassette is integrated into an expression vector.

49. A method of determining the specific activity of a cloned 11 alpha

hydroxylase comprising the steps of;

- (a) transforming host cells with an expression vector comprising a nucleic acid that encodes said 11 alpha hydroxylase,
- (b) expressing said 11 alpha hydroxylase in said host cells;
- 5 (c) preparing subcellular membrane fractions from said cells,
- (d) incubating said subcellular membrane fractions microsomes with a steroid substrate, and
- (e) monitoring conversion of the steroid substrate to its 11 alpha hydroxy steroid counterpart.

10 50. An isolated and purified antibody having a binding specificity for 11 alpha hydroxylase of claim 3 as shown in SEQ ID NO: 2.

51. An isolated and purified antibody having a binding specificity for the 11 alpha hydroxylase of claim 4.

15 52. The antibody of claim 51 which binds to a protein region selected from the group consisting of

- (a) the N-terminal amino acids 1-10 of SEQ ID NO: 2;
- (b) the last 10 C-terminal amino acids of SEQ ID NO: 2;
- (c) SEQ ID NO: 23;
- (d) SEQ ID NO: 24; and
- (e) SEQ ID NO: 25.

20 53. An isolated and purified antibody having a binding specificity for oxidoreductase of claim 8

54. An isolated and purified antibody having a binding specificity for the oxidoreductase of claim 9.

25 55. The antibody of claim 54 which binds to a protein region selected from the group consisting of:

- (a) the N-terminal amino acids 1-10 of SEQ ID NO: 6;

(b) the last 10 C-terminal amino acids of SEQ ID NO: 6; and

(c) SEQ ID NO: 26.

56. A composition comprising the antibody of claim 50 or 53 and an effective carrier, vehicle, or auxiliary agent.

5 57. An isolated nucleic acid that specifically hybridizes under low stringent conditions to the nucleic acid of claim 2

58. An isolated nucleic acid that specifically hybridizes under high stringent conditions to the nucleic acid of claim 2.

10 59. An isolated nucleic acid that specifically hybridizes under low stringent conditions to the nucleic acid of claim 7

60. An isolated nucleic acid that specifically hybridizes under high stringent conditions to the nucleic acid of claim 7.

15 61. Use of a host cell harboring a cloned 11 alpha hydroxylase of claim 1 for the manufacture of a medicament for therapeutic application to treat heart disease, inflammation, arthritis, or cancer.

62. A composition comprising from about 0.5-500 g/L molasses, 0.5-50 g/L cornsteep liquid, 0.5-50 g/L  $\text{KH}_2\text{PO}_4$ , 2.5-250 g/L NaCl, 2.5-250 g/L glucose, and 0.04-4 g/L progesterone, pH 3.5-7.

20 63. The composition of claim 62 comprising from about 10-250 g/L molasses, 1-25 g/L cornsteep liquid, 1-25 g/L  $\text{KH}_2\text{PO}_4$ , 5-125 g/L NaCl, 5-125 g/L glucose, and 0.08-2 g/L progesterone, pH 4.5-6.5.

64. The composition of claim 62 comprising from about 25-100 g/L molasses, 2.5-10 g/L cornsteep liquid, 2.5-10 g/L  $\text{KH}_2\text{PO}_4$ , 12.5-50 g/L NaCl, 12.5-50 g/L glucose, and 0.2-0.8 g/L progesterone, pH 5.5-6.0.

25 65. The composition of claim 62 comprising from 50 g/L molasses, 5 g/L cornsteep liquid, 5 g/L  $\text{KH}_2\text{PO}_4$ , 25 g/L NaCl, 25 g/L glucose, 20 g/L agar, and 0.4 g/L progesterone, pH 5.8.

66. A composition of claim 62 further comprising from about 4-100 g/L agar.

67. A composition of claim 66 further comprising from about 10-40 g/L agar.

68. A composition of claim 66 further comprising about 20 g/L agar.

69. Use of the composition of claim 62 or 66 to produce spores from the microorganism selected from the group consisting of *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus nidulans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, and *Trichothecium roseum*, *Fusarium oxysporum* *Rhizopus arrhizus*, *Monosporium olivaceum*, *Penicillium chrysogenum*, and *Absidia coerulea*.

70. Use of the composition of any of claims 62 or 66 to produce spores from *Aspergillus ochraceus*.

71. A fusion protein comprising the amino acid sequence of the *Aspergillus ochraceus* oxidoreductase of claim 8.

72. An isolated and purified *Aspergillus ochraceus* 11 alpha hydroxylase encoded by the nucleic acid of claim 57.

73. An isolated and purified *Aspergillus ochraceus* 11 alpha hydroxylase encoded by the nucleic acid of claim 58.

74. An isolated and purified *Aspergillus ochraceus* oxidoreductase encoded by the nucleic acid of claim 59.

75. An isolated and purified *Aspergillus ochraceus* oxidoreductase encoded by the nucleic acid of claim 60.

76. A method of converting a steroid to its 11 alpha hydroxylated form comprising the steps of:

a.) contacting the steroid with the *Aspergillus ochraceus* 11 alpha hydroxylase of claim 3

77. A 11 alpha hydroxylated steroid made by the method of claim 76.